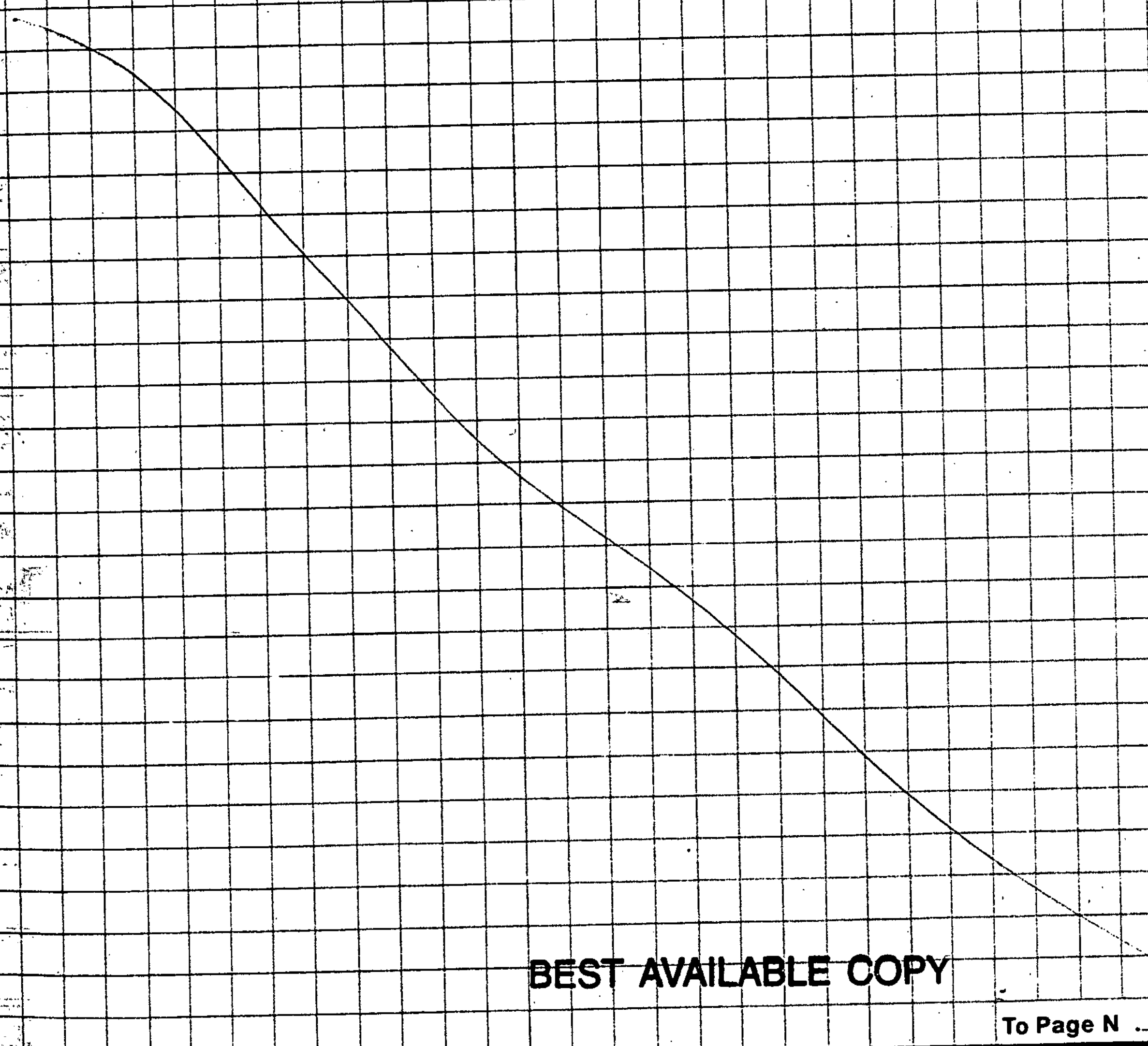
Project No.________ Figure A

TITLE Control Cells: Redundant Dual Staining Book No.________

From Page No.__ Objective: concept proposal for dual labeling for improve the probability of stained control cells (See attachment from Fig [illegible]/00 showing statistics of non-stained control cells at 200 and 1000 cells [illegible]. With dual staining these probabilities would be x^2 rather than x.

The rationale and concept are specific types are outlined in my memo of [illegible]/00 (attached)

The idea may be patentable.



BEST AVAILABLE COPY

To Page N ______

Witnessed & Understood by me, [signature] Date 9/21/0[illegible]

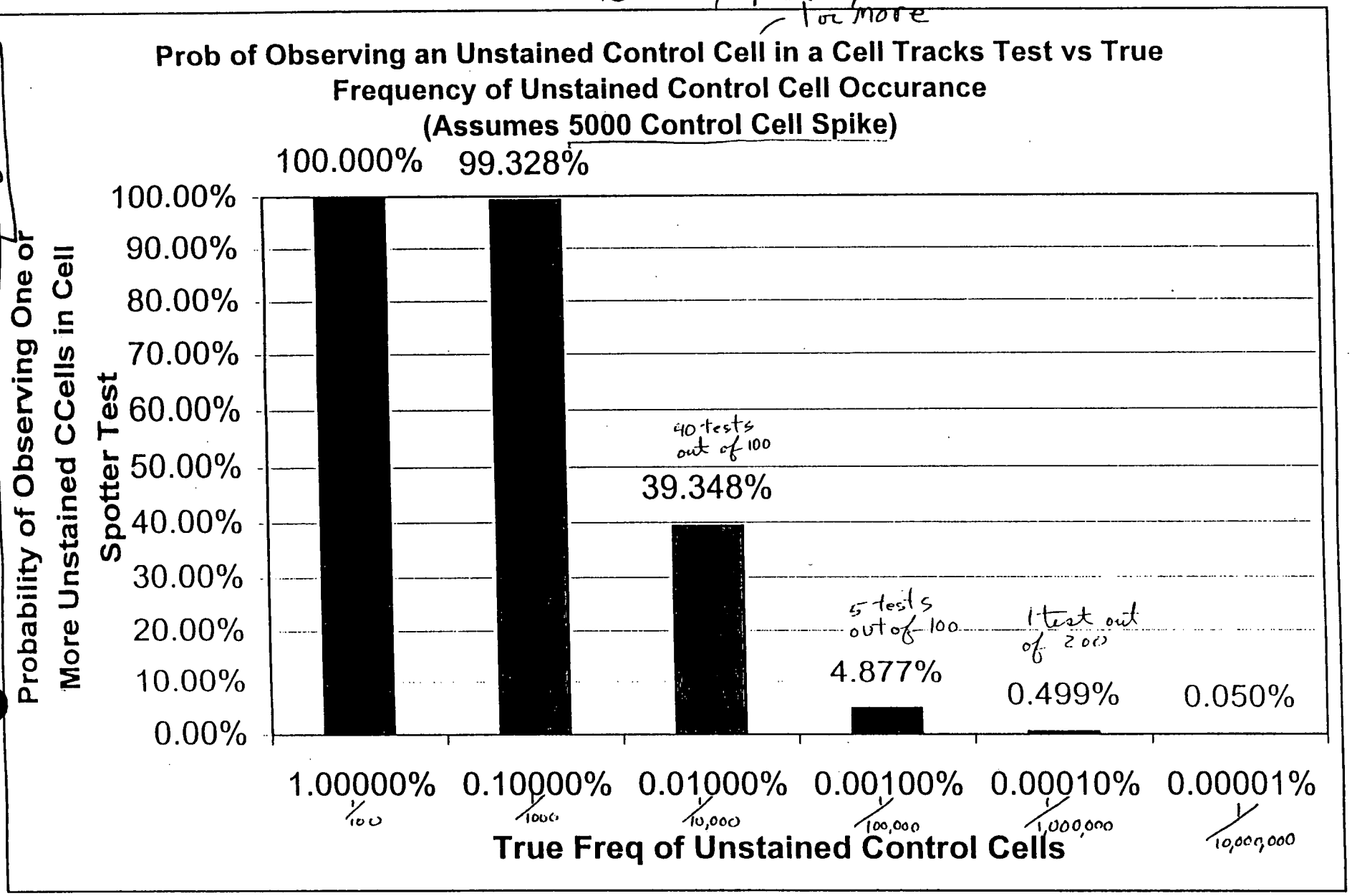
Invented by [signature] Date [illegible]/00

Recorded by

Figure B

a. Leon 4/24/00
Gerry
Tom, Paul, DiG
for more

32C-8C



TdM
5/24/2000

Figure C

04/21/2000
TO: Leon
CC. Tom, Tim, Mike K., Gerry D., Chandra, DIG, John

Subject: Additional or Redundant Stain for Control Cells

Recent concerns about potential misclassification of control cells as tumor cells suggest the need for greater certainty in identifying differentially stained control cells to be added to patient specimens at a level of 1000-5000 cells. The current control cells, permeabilized/fixed SKBR3 cells, are stained with the unique membrane fluor, DiOC16 (3) that emits strongly in the green channel. The control cells are further identified by the generic nucleated cell stain, DAPI and C-11 PE, i.e. control cells are visible in filters #1, #2 and #3 and invisible in filter #4.

Staining of control cells by the recently improved process has shown essentially complete staining of ALL cells in a population of 5000 cells, consistently showing zero unstained cells per 5000 stained cells by flow analysis (MH data). However, among millions of cells in a given lot there may indeed be a few unlabeled or weakly labeled cells that could be misclassified as tumor cells in a patient specimen. A second unique or independent discriminant on the control cells would further reduce the compound probability of having unstained or weakly DiOC-stained control cells to essentially zero.

The second unique stain could be another fluor, either:
1. A specific nuclear stain or
2. A specific cell surface stain neither of which should not interfere with emissions from the other stains: DAPI, APC and PE.

It must also excite at or near one of the four excitation wavelengths, 365, 480, 546 or 620nm, show no bleed-through into adjacent channels and must not overwhelm the signals from the other stains.

A. Candidates for Option #1 (nuclear staining):

1. Ethidium bromide, 493nm/620nm (for dsDNA); excitation in the green channel (#2) emission in the yellow channel (#3). Prestaining of permeabilized fixed control cells with EB could be done partially to permit additional nuclear staining with DAPI in the test protocol (dual nuclear staining).

Pros: The control cells would show signals in channels #1 (DAPI), #2 (DiOC16 (3)) and #3(PE). Since DiOC16 (3) staining is normally very intense, the EB nuclear stain in channel #2 would probably be masked in most control cells. However, the nuclear stain would be more clearly visible in weakly stained or unstained control cells. EB staining would thus complement DiOC staining. This dual specific staining of the control cells would considerably reduce the probability of having an unstained control cell. No licensing is needed.
Cons: EB staining would have to be optimized so as not to interfere with DAPI staining.

EB is somewhat light sensitive, but the DiOC in the membrane may shield it from exposure to ambient light during storage. FF-bound DNA may show as debris in channel #3.

2. Acridine Orange: 503nm/530nm (dsDNA). This nuclear stain excites and emits in the green channel like DiOC and would normally be totally masked by DiOC. However, in control cells, weakly stained or unstained, the presence of a green nucleus would confirm the identity of the control cell. Hence AO would be a backup or supplemental marker to DiOC staining in the SAME channel. No licensing is needed.

3. YO-PRO-1: 491nm/509nm. This dye should behave similar to AO. Licensing may be required.

B. Candidates for Option #2 (cell surface staining):

1. Dansyl chloride: 372nm/520nm. Reacts covalently with surface amino groups from proteins on non-permeabilized/unfixed cells to form stable sulfonamide linkages. The attached dye would mimic a membrane stain. Excitation would occur along with DAPI in channel #1, but emission would be in channel #2. Dansyl emission would be seen in the same channel as DiOC and would confirm a control cell even if channel #2 shows weak or absent DiOC emission. Labeling BEFORE permeabilization may be needed to prevent labeling of internal cell components that could interfere with C-11 PE binding. Good photostability; no licensing needed.
   Con: Dansyl chloride reacts with surface amino groups of proteins and may interfere with binding of capture antibody.

2. FITC, 495nm/519nm, or similar reactive fluors would also react with surface amino groups and could be used as a backup for DiOC with excitation and emission in the DiOC channel. Emissions may only be visible in the absence of DiOC. No licensing needed. Photostability is poor. Other reactive dyes from M.P. are more photostabile but would require licensing.

Some of these proposals could be tested with existing DiOC labeled cells. All would require minor process optimization or changes.

The proposed concept of "backup" or redundant labeling of control cells should improve acceptance by the FDA and other skeptics who may question the rationale of adding stained tumor cells to specimens for process control purposes.

HR
P.S. Based on feedback from Leon, the proposed dyes may not be satisfactory. However, the proposal appears conceptually sound.